EFFECTS OF ACTIVATION PEPTIDE BOND CLEAVAGE AND FRAGMENT 2 INTERACTIONS ON THE PATHWAY OF EXOSITE I EXPRESSION DURING ACTIVATION OF HUMAN PRETHROMBIN 1 TO THROMBIN*

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Running title: Formation of Regulatory Exosite I on Thrombin
Summary

Activation of prothrombin (Pro) by factor Xa to form thrombin occurs by proteolysis of Arg$^{271}$-Thr$^{272}$ and Arg$^{320}$-Ile$^{321}$, resulting in expression of regulatory exosites I and II. Cleavage of Pro by thrombin liberates fragment 1 and generates the zymogen analog, prethrombin 1 (Pre 1). The properties of exosite I on Pre 1 and its factor Xa activation intermediates were characterized in spectroscopic and equilibrium binding studies using the fluorescein-labeled probe, hirudin$^{54-65}$ ([5F]Hir$^{54-65}$ (SO$_3^-$)). Prethrombin 2 (Pre 2), formed by factor Xa cleavage of Pre 1 at Arg$^{271}$-Thr$^{272}$, had the same affinity for hirudin$^{54-65}$ peptides as Pre 1 in the absence or presence of near-saturating fragment 2 (F2). Pre 2 and thrombin also had indistinguishable affinities for F2. By contrast, cleavage of Pre 1 at Arg$^{320}$-Ile$^{321}$, to form active meizothrombin des-fragment 1 MzT(-F1), showed a 11-20 fold increase in affinity for hirudin$^{54-65}$, indistinguishable from the 13-20 fold increase seen for conversion of Pre 2 to thrombin. Thus, factor Xa cleavage of Pre 1 at Arg$^{271}$-Thr$^{272}$ does not effect exosite I expression, whereas cleavage at Arg$^{320}$-Ile$^{321}$ results in concomitant activation of the catalytic site and exosite I.

Furthermore, expression of exosite I on the Pre 1 activation intermediates is not modulated by F2 and exosite II is not activated conformationally. The differential expression of exosite I affinity on the Pre 1 activation intermediates and the previously demonstrated role of (pro)exosite I in factor Va-dependent substrate recognition suggest that changes in exosite I expression may regulate the rate and direction of the Pre 1 activation pathway.
Activation of prothrombin (Pro) to form the serine proteinase, thrombin, is the central zymogen activation reaction in the blood coagulation system (1, 2). Pro is activated by factor Xa cleavage of Arg\textsuperscript{271}-Thr\textsuperscript{272}, between the fragment 2 (F2) and catalytic (prethrombin 2, Pre 2) domains, and Arg\textsuperscript{320}-Ile\textsuperscript{321}, within the catalytic domain (3, 4). A 300,000-fold rate acceleration of Pro activation is regulated by assembly interactions among the protein cofactor, factor Va, phospholipid membranes, and calcium (2, 5-7). Factor Va regulates the rate and pathway of Pro activation through a mechanism that is not clearly understood. The protein cofactor alters the specificity of factor Xa for peptide bond cleavage in Pro, with the catalytically active intermediate meizothrombin (MzT) accumulating preferentially (8-11). In the absence of the cofactor, cleavage of Arg\textsuperscript{271}-Thr\textsuperscript{272} results in accumulation of the inactive precursor, Pre 2 associated with activation fragment 1.2, with only traces of MzT accumulating.

Once formed, the activity of thrombin for physiological substrates and regulatory macromolecules is controlled by interactions mediated by exosites I and II on the enzyme surface. The critical roles of exosites I and II in regulation of macromolecular interactions of thrombin are well established (12, 13), including the role of exosite I in fibrinogen substrate recognition (14,15), and both exosites in thrombomodulin-regulated protein C activation (16-18), factor V (19-22) and factor VIII (19) activation. In addition, exosite II binds heparin (23), regulating inactivation of thrombin by antithrombin (24), and Pro fragment 2 (F2, 25). On Pro, exosite I is in a precursor, low affinity state (proexosite I) that is activated on conversion of Pro to thrombin, as shown by a ~100-fold increase in affinity for an exosite I-specific hirudin\textsuperscript{54-65} peptide probe (26). Characterization of proexosite I supports an important a role for this site in factor Va-
mediated Pro binding and recognition as a substrate of the factor Xa-factor Va catalytic complex (27-29). Recent mutagenesis studies in which proexosite I residues were shown to play a critical role in factor Va acceleration of prethrombin 1 (Pre 1) activation support the importance of this site in substrate recognition (30). In Pro and Pre 1, exosite II is covered by the covalently linked fragment 2 domain (25). Whether exosite II is expressed as a result of passive dissociation of F2 following cleavage at Arg$^{271}$-Thr$^{272}$ or by a conformational change affecting affinity for F2 is not known. Delineating the pathway of expression of exosites I and II is important to understanding the roles of these sites in the mechanism of factor Va acceleration of Pro activation, regulation of the activation pathway, and regulation of physiological interactions of the Pro activation intermediates and thrombin.

Previous studies of exosite I interactions of Pro activation species do not provide a consistent view of the pathway of expression of exosite I. Exosite I was accessible on human meizothrombin des-fragment 1 (MzT(-F1)) for binding of thrombomodulin and an anti-exosite I antibody, whereas exosite I on human Pro, Pre 2, and MzT did not bind these macromolecules (31). In studies of the bovine proteins, however, binding of fluorescein-labeled hirudin$^{53-64}$ (hirugen) to Pro and Pre 1 was undetectable, while Pre 2 bound the peptide with similar affinity as thrombin (32). MzT and MzT(-F1) both bound the peptide with 2-8 fold higher affinity than Pre 2, suggesting that either peptide bond cleavage in Pro results in activation of exosite I (32). Comparison of the previous studies is difficult because of the use of different ligands and proteins from different species. This prompted a quantitative analysis of the pathway of expression of exosite I
on the human Pro activation intermediates and investigation of the effects of the activation fragments on exosite I affinity.

Pre 1, formed by removal of fragment 1 (F1) from Pro by thrombin cleavage at Arg^{155}-Ser^{156}, and the Pre 1 activation intermediates, Pre 2 and F2, and MzT(-F1), were chosen as analogs of Pro for initial investigation of the expression of exosites I and II and the influence of F2. Hirudin^{54-65} fluorescently labeled with 5-carboxyfluorescein ([5F]Hir^{54-65}(SO_3^-)) was used as an exosite I-specific probe in equilibrium binding studies to characterize the properties of exosite I on the Pre 1 species in comparison with previous results for Pro and thrombin (26). Conversion of Pro to Pre 1 was accompanied by a surprising 2-6 fold increase in affinity of Pre 1 for hirudin^{54-65} peptides. Among Pre 1 activation intermediates, Pre 2 bound the peptide with the same affinity as Pre 1, whereas MzT(-F1) displayed a 11-20 fold increase in affinity, with a dissociation constant indistinguishable from that determined for thrombin. F2 bound with similar affinity to Pre 2 and thrombin, indicating no change in affinity of exosite II for this specific ligand accompanying the proteinase activating conformational change. The results demonstrate that proteolytic cleavage of Pre 1 in the proteinase zymogen domain by factor Xa results in activation of regulatory exosite I as well as the catalytic site, whereas cleavage at Arg^{271}-Thr^{272} does not change exosite I affinity significantly. The differential expression of exosite I affinity on the Pre 1 activation intermediates is thought to play a role in the activity of factor Va to regulate the rate and direction of the Pre 1 activation pathway. In the companion paper (33), these studies are extended to the Pro activation intermediates to investigate the effect of the F1 domain in modulating activation of exosite I.
EXPERIMENTAL PROCEDURES

Protein purification and characterization- Human Pro, factor X, factor Xa, and thrombin were purified and characterized as described previously (34, 35). Thrombin was active-site labeled with $N^\alpha$-[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH$_2$Cl (ATA-FPR-CH$_2$Cl) and 5- or 6-(iodoacetamido)fluorescein, and the resulting [5F]FPR-thrombin and [6F]FPR-thrombin were characterized as described previously (34, 36). Pre 1 and F1 were prepared by cleavage of Pro with thrombin (34). Pre 1 was activated with partially-purified ecarin from Echis carinatus venom in the presence of a 3-fold excess ATA-FPR-CH$_2$Cl to generate ATA-FPR-MzT(-F1) (37). Pre 2 and F2 were prepared from factor Xa activation reactions and separated by a modification of the published method (11). Pre 1 (100 mg) was incubated with 0.4 µM factor Xa for 90 min in 100 mM Hepes, 10 mM CaCl$_2$, pH 7.6, at 25 °C in the presence of 10 µM Thromstop (American Diagnostica). The reaction was quenched by immediate loading onto soybean trypsin inhibitor-agarose (1.5 cm x 24 cm) at 4 °C. Pre 2 and F2 were eluted from the column in 50 mM Hepes, 110 mM NaCl, 5 mM CaCl$_2$, pH 7.4. Fractions were collected into 10 mM MES, 1 mM EDTA, 10 mM benzamidine, pH 6.0 to prevent slow proteolysis. The eluted proteins were loaded immediately onto Blue-Sepharose (2.5 cm x 14 cm) at 4 °C, equilibrated with 50 mM Tris, 0.1 M NaCl, 1 mM benzamidine, 1 mM EDTA, 0.02% NaN$_3$, pH 7.5. F2 eluted from the column in the wash fractions, while Pre 2 bound to the column and was eluted with a 1 L gradient of buffer containing up to 1.5 M NaCl. Pre 2 was purified further by chromatography on benzamidine-Sepharose (1.5 cm x 28 cm) to remove thrombin (11). Pre 2 was concentrated, dialyzed against 50 mM Hepes, 0.125 M NaCl, pH 7.4, snap frozen, and stored at -80 °C. Protein concentrations were
determined by absorbance at 280 nm with the following absorption coefficients ((mg/ml\(^{-1}\)cm\(^{-1}\)) and molecular weights (8, 38, 39): Pro, 1.47, 71,600; Pre 1, 1.78, 49,900; Pre 2, 1.73, 37,000; MzT(-F1), 1.78, 49,900; thrombin, 1.74, 36,600; F1, 1.19, 21,700; F2, 1.42, 12,800.

Hir\(^{54-65}(SO_3^-)\) (Sigma or Bachem) was dissolved in water or buffer and the concentration was determined by the purity and peptide content specified by the manufacturer. The amino-terminus of Hir\(^{54-65}(SO_3^-)\) was labeled with 5’-carboxy(fluorescein) ([5F]Hir\(^{54-65}(SO_3^-)\)) as described previously (40).

Quantitative affinity chromatography - Hir\(^{54-65}(SO_3^-)\) binding to Pre 1 was analyzed by quantitative affinity chromatography on Hir\(^{54-65}(SO_3^-)\)-agarose. Hir\(^{54-65}(SO_3^-)\) was coupled to Affigel-10 (BioRad) as described previously (26), except that 0.2 mg of peptide was coupled to ~3 ml of gel. Chromatography experiments were performed by loading 300 \(\mu\)l of 6.7 \(\mu\)M Pre 1 (0.1 mg) in 50 mM Hepes, 110 mM NaCl, 5 mM CaCl\(_2\), 1 mg/ml polyethylene glycol 8000, pH 7.4 onto 2.7 ml columns at a flow rate of 1.5 ml/hr. Pre 1 was preincubated for 30 min at 25 °C with 1 \(\mu\)M FPR-CH\(_2\)Cl and various concentrations of Hir\(^{54-65}(SO_3^-)\) prior to chromatography. The elution volume (\(V_{obs}\)) was determined from the midpoint of the \(A_{230\,nm}\)-absorbance peak. The recovery of the protein eluted in the separations was estimated to be 40-108% by integration of the peaks. Pre 1 was also subjected to chromatography on a control matrix prepared without peptide to determine the elution volume under conditions where the protein did not interact with the gel (\(V_o\)), and similarly in the presence of buffer containing 2 M NaCl. A concentration of \(\geq81\) \(\mu\)M binding sites for Pre 1 on the gel matrix was determined by batchwise titrations of 50 \(\mu\)l of gel with Pre 1 and measurement of
binding from the decrease in solution protein absorbance. This confirmed that under the chromatography conditions only a small fraction of the matrix binding sites was occupied. On this basis, the dependence of the fractional change in elution volume \(((V_{obs} - V_0)/V_0)\) on the total Hir\(^{54-65}(SO_3^-)\) concentration was analyzed using the hyperbolic equation (26, 41, 42) to determine the dissociation constant for Hir\(^{54-65}(SO_3^-)\) binding to Pre 1 in solution and the ratio of the total concentration of matrix sites to the dissociation constant for matrix binding.

**Fluorescence studies**- Fluorescence intensities were measured with an SLM 8100 fluorometer in acrylic cuvettes coated with polyethylene glycol 20,000. All experiments were performed in 50 mM Hepes, 110 mM NaCl, 5 mM CaCl\(_2\), 1 mg/ml polyethylene glycol 8000, pH 7.4, and at 25 °C. Experiments with the Pre 1 activation intermediates contained 0.1 µM FPR-CH\(_2\)Cl. Fluorescence excitation spectra of 0.2 µM [5F]Hir\(^{54-65}(SO_3^-)\) with near-saturating concentrations of Pre 1 activation species were recorded at the emission maximum of 520 nm (4 nm excitation and emission band passes). Spectra were normalized to the initial fluorescence intensity of [5F]Hir\(^{54-65}(SO_3^-)\) measured with excitation at 491 nm. Corrections for background (≤1%) were made from parallel measurements on blanks lacking the labeled peptide, and corrections for dilution were ≤5%.

**Binding of hirudin peptides to Pre 1 and the activation intermediates**- Binding of [5F]Hir\(^{54-65}(SO_3^-)\) was measured by titrating the labeled peptide with each protein and monitoring the change in fluorescence at 520 nm with excitation at 491 nm. Fluorescence changes expressed as \((F_{obs} - F_0)/F_0 = \Delta F/F_0\) as a function of total protein concentration were fit by the quadratic binding equation to obtain the maximum
fluorescence change \((F_{\text{max}} - F_0)/F_0 = \Delta F_{\text{max}}/F_0\) and the dissociation constant \((K_D)\). For MzT(-F1), the stoichiometry \((n)\) was also obtained, while for Pre 1 and Pre 2, \(n\) was fixed at 1.

Competitive binding of unlabeled peptide and \([5F]\text{Hir}^{54-65}(SO_3^-)\) was quantitated in titrations of mixtures of fixed concentrations of probe and protein with the unlabeled peptide. The return of the observed fluorescence change toward the starting value was measured as a function of unlabeled peptide concentration. The data were corrected for a small linear increase (<2.4 %/µM Hir54-65(SO3^-)) in fluorescence of \([5F]\text{Hir}^{54-65}(SO_3^-)\) when titrated with unlabeled peptide. Together with the direct titrations, the competitive displacement data were fit simultaneously by the cubic equation for competitive binding to determine the maximal fluorescence change and the dissociation constant for the unlabeled peptide interaction, with \(n\) fixed at 1 (43, 44).

**Binding of F2 to thrombin and Pre 2**- Binding of F2 to thrombin was determined by monitoring the change in fluorescence of 190 nM [6F]FPR- or [5F]FPR-thrombin as a function of F2 concentration. Competitive binding of F2 to Pre 2 and thrombin was measured by titration with F2 of mixtures of 190 nM [6F]FPR- or [5F]FPR-thrombin and 20 µM Pre 2. The titrations were fit by the cubic equation for competitive binding to obtain \(\Delta F_{\text{max}}/F_0\) and the dissociation constant for F2 binding to either the labeled thrombin or Pre 2 with \(n=1\) (43, 44).

**Effect of F2 on binding of hirudin peptides to Pre 2**- The effects of F2 on binding of \([5F]\text{Hir}^{54-65}(SO_3^-)\) to Pre 2 were determined by titration of 50 nM \([5F]\text{Hir}^{54-65}(SO_3^-)\) and 20 µM F2 with Pre 2 and compared to similar titrations in the absence of F2. Scheme I describes the random addition ternary complex model for F2 and \([5F]\text{Hir}^{54-65}(SO_3^-)\) (H).
binding to Pre 2 (P2) to form the binary complexes, P2·F2 or P2·H, and addition of the alternate ligand to the binary complexes to form the ternary complex, P2·F2·H. The model was used to analyze the titrations of [5F]Hir^{54-65}(SO_3^-) with Pre 2 in the presence and absence of F2 to determine the dissociation constants for [5F]Hir^{54-65}(SO_3^-) binding to Pre 2 (K_{P2(H)}), F2 binding to Pre 2 (K_{P2(F2)}), and the formation of the ternary complex, P2·F2·H (K_{P2F2(H)} or K_{P2H(F2)}):

\[ K_{P2(H)} \quad \quad \quad K_{P2F2(H)} \]
\[ P2 + F2 + H \quad \quad \quad \quad \quad P2·F2·H \]
\[ K_{P2(F2)} \quad \quad \quad K_{P2F2(H)} \]

Scheme I

The observed fluorescence changes of the labeled hirudin peptide in Scheme I can be described as a function of the concentration of the two fluorescent complex species relative to probe alone, as given by:

\[ \frac{\Delta F}{F_0} = \frac{\Delta F_{\text{max } P2(H)}}{F_0} \left( \frac{[P2 \cdot H]}{[H]^b} \right) + \frac{\Delta F_{\text{max } P2F2(H)}}{F_0} \left( \frac{[P2 \cdot F2 \cdot H]}{[H]^b} \right) \]

Computer solution of the equations for the dissociation constants and mass conservation was used to determine the concentration of the binary complex, [P2·H], and the concentration of the ternary complex, [P2·F2·H], the dissociation constants for [5F]Hir^{54-65}(SO_3^-) binding to Pre 2 in the absence and presence of F2 (K_{P2(H)} and K_{P2F2(H)}), and the maximum fluorescence changes for each of the fluorescent complexes.
(ΔF_{max} P2(H)/F_0 and ΔF_{max} P2F2(H)/F_0). The dissociation constant for F2 binding to Pre 2
(K_{P2(F2)}) was fixed at the value determined previously as described above.

Nonlinear least-squares fitting was performed with Scientist software. Error
estimates represent ±2 SD.
RESULTS

Fluorescence spectral properties of [5F]Hir$^{54-65}$($\text{SO}_3^-$) binding to prethrombin 1, prethrombin 1 activation intermediates, and thrombin- Spectral changes accompanying binding of fluorescein-labeled hirudin$^{54-65}$ were evaluated from excitation difference spectra and from the maximum amplitudes of the spectral changes determined in titrations as described below. Upon [5F]Hir$^{54-65}$($\text{SO}_3^-$) binding to Pre 1 and the Pre 1 activation intermediates, the fluorescence excitation difference spectra showed distinct features that differentiated the characteristics of exosite I on Pre 1 from those on the activation intermediates (Fig. 1). Pre 1 quenched the fluorescence of [5F]Hir$^{54-65}$($\text{SO}_3^-$) by $21.1 \pm 0.5\%$. The S-shaped difference spectrum was similar to that seen previously for the same peptide binding to Pro but the fluorescence change was slightly (3%) larger (Table I, 26). The fluorescence excitation difference spectra for [5F]Hir$^{54-65}$($\text{SO}_3^-$) binding to Pre 2 exhibited properties similar to that of Pre 1, with the fluorescence quenched $26.5 \pm 0.5\%$. By contrast, the spectra for the fluorescein-labeled peptide binding to MzT(-F1) displayed characteristics that were essentially identical to those of thrombin (Fig. 1), where the fluorescence was quenched by $29.8 \pm 0.5$ and $29.2 \pm 0.4\%$, respectively (Table I, 26). Comparison of the excitation difference spectra and the maximum amplitudes suggested that exosite I on Pro, Pre 1, and Pre 2 was slightly different, while exosite I on MzT(-F1) and thrombin was the same. This suggested slightly different states of exosite I for the inactive zymogen precursors, distinctly different from the environment on the catalytically active products.

Binding of hirudin peptides to prethrombin 1- The affinity of Hir$^{54-65}$($\text{SO}_3^-$) for Pre 1 was determined initially by small-zone quantitative affinity chromatography. Pre 1 eluted
from Hir$^{54-65}$(SO$_3^-$)-agarose as a broad peak in 0.15 M, 5 mM CaCl$_2$, pH 7.4 buffer and was eluted in successively smaller volumes from the column equilibrated with increasing concentrations of free peptide (Fig. 2). Pre 1 eluted in the void volume of the peptide column in 2 M NaCl and a control column lacking peptide, indicating a specific interaction of Pre 1 with the immobilized peptide. Analysis of the hyperbolic decrease in elution volume of Pre 1 as a function of increasing peptide concentration gave a dissociation constant of 0.7±0.1 µM for Hir$^{54-65}$(SO$_3^-$) binding to Pre 1, with an intercept of 6.2 ± 0.2 for the ratio of the concentration of matrix sites to the dissociation constant for matrix binding (Fig. 2, *inset*). The latter result was reasonably consistent with the independently determined value of 3.1 from titration of the gel matrix with Pre 1 (see “Experimental Procedures”). The results showed that Pre 1 bound Hir$^{54-65}$(SO$_3^-$) with 5-fold higher affinity in comparison to Pro (Table I) and demonstrated that the enhanced affinity was a bulk property of Pre 1 and not due to contaminating proteins that bound with higher affinity.

Exosite I on Pre 1 was characterized further in fluorescence equilibrium binding experiments. Addition of increasing concentrations of Pre 1 to [5F]Hir$^{54-65}$(SO$_3^-$) quenched the fluorescein fluorescence (Fig. 3A) with a dissociation constant of 0.5±0.1 µM, which was in very good agreement with the value determined by chromatography. This represented a 6-fold increase in affinity of the peptide for Pre 1 compared to the dissociation constant previously determined for Pro of 3.2±0.3 µM (Table 1, 26).

Titration with Pre 1 in the presence of 30 µM F1 produced no change in affinity for the labeled peptide, consistent with no direct interaction of free F1. Competitive titrations of [5F]Hir$^{54-65}$(SO$_3^-$) binding with the unlabeled peptide, Hir$^{54-65}$(SO$_3^-$), confirmed the 4-6
fold higher affinity of the peptide for Pre 1 compared to Pro (Fig. 3B, Table I). The results suggested that removal of F1 either caused a conformational change in exosite I on Pre 1 that increased affinity for hirudin peptides, or that the F1 domain on Pro interfered with peptide binding to exosite I. This observation is addressed further in the companion manuscript (33).

*Binding of fragment 2 to [6F]FPR-thrombin, [5F]FPR-thrombin, and prethrombin 2-*

Interactions of F2 with exosite II on thrombin and Pre 2 were characterized in equilibrium binding experiments with thrombin labeled at the active site with 6-(iodoacetamido)fluorescein ([6F]FPR-thrombin) or 5-(iodoacetamido)fluorescein ([5F]FPR-thrombin). F2 quenched the fluorescence of [6F]FPR-thrombin by 26±2% and bound with a dissociation constant of 7±2 µM (Fig. 4), essentially the same as previously determined (37, 45). Competitive titrations of F2 binding to labeled thrombin and unlabeled Pre 2 gave a dissociation constant of 3±1 µM for F2 binding to Pre 2, which represented a small (~2-fold) and probably insignificantly higher affinity for Pre 2 in comparison to thrombin (Fig. 4). Titrations of [5F]FPR-thrombin with F2 produced similar results, with a dissociation constant of 13±3 µM and a 13±1% quench of the fluorescence (data not shown). Competitive titrations of F2 binding to [5F]FPR-thrombin and unlabeled Pre 2 gave a dissociation constant of 7±3 µM for F2 binding to Pre 2, confirming a ≤2-fold higher affinity of F2 for Pre 2 in comparison to thrombin (data not shown). These results indicated little or no change in exosite II affinity for F2 upon conversion of Pre 2 to thrombin.

*Binding of hirudin peptides to prethrombin 2 and the prethrombin 2-fragment 2 complex-*

Characterization of exosite I on Pre 2 by direct titration of [5F]Hir\textsuperscript{54-65}(SO\textsubscript{3}\textsuperscript{−}) resulted in
the same dissociation constant as obtained for Pre 1 (Fig. 5A, Table 1). Binding of unlabeled Hir$_{54-65}$(SO$_3^-$) to Pre 2 evaluated in competitive titrations with the labeled peptide yielded the same $K_D$ for the peptides (Fig. 5B). Similar titrations of the labeled peptide with Pre 2 were performed in the presence of near-saturating F2 (20 µM). Analysis of this data by a ternary complex model (Scheme I, “Experimental Procedures”) gave indistinguishable dissociation constants for the binary Pre 2·[5F]Hir$_{54-65}$(SO$_3^-$) complex ($K_{P2(H)} 0.43±0.04$ µM) and the ternary Pre 2·F2·[5F]Hir$_{54-65}$(SO$_3^-$) complex ($K_{P2F2(H)} 0.4±0.1$ µM). The model also yielded indistinguishable maximum fluorescence changes for Pre 2·H (-26.5±0.6 %) and Pre 2·F2·H (-25.8±0.7 %) (Fig. 5A, Table 1). The results showed no detectable change in exosite I affinity upon cleavage of Pre 1 at Arg$^{271}$-Thr$^{272}$ to form Pre 2. Binding of F2 to Pre 2 did not alter peptide binding to exosite I, indicating no detectable thermodynamic linkage between exosite I and II interactions with Pre 2.

**Binding of hirudin peptides to meizothrombin des-fragment 1- Exosite I**

Exosite I on the catalytically active intermediate, MzT(-F1), was characterized by a 20-fold higher affinity for [5F]Hir$_{54-65}$(SO$_3^-$) ($K_D 24±3$ nM) compared to Pre 1 and Pre 2 (Fig. 6A, Table 1). Competitive titrations of labeled and unlabeled Hir$_{54-65}$(SO$_3^-$) with MzT(-F1) showed a similar dissociation constant of 65±13 nM for the unlabeled peptide (Fig. 6B). The affinity of the labeled peptide for MzT(-F1) was indistinguishable from that for thrombin (Table I). These results showed that cleavage of Pre 1 at Arg$^{320}$-Ile$^{321}$ altered the conformation of exosite I such that it bound the hirudin peptides with 11-20 fold increased affinity. Cleavage of Arg$^{320}$-Ile$^{321}$ in the proteinase domain of Pre 1 resulted in simultaneous activation of exosite I and the proteinase catalytic site, whereas no
change in exosite I was seen with cleavage of Pre 1 at Arg^{271}-Thr^{272} between the proteinase and F2 domains.
DISCUSSION

These studies characterized quantitatively the pathway of exosite activation on human Pre 1 activation intermediates for the first time. Pre 1 was used as an analog of Pro to simplify characterization of the effects of activation peptide bond cleavages on the expression of exosites I and II. Fluorescence excitation spectra of $[5F]{\text{Hir}}^{54-65}({\text{SO}}_3^{-})$ bound to Pro, Pre 1, the Pre 1 activation intermediates, and thrombin suggested small differences between the exosite environments on Pro, Pre 1, and Pre 2 zymogen species, distinct from the indistinguishable environment of the peptide-probe bound to exosite I on the catalytically active forms. All but one of the spectral changes were associated with changes in affinity of exosite I on the Pre 1 activation species. Cleavage of Pre 1 by factor Xa at Arg$^{271}$-Thr$^{272}$, to yield Pre 2 and F2, resulted in no detectable change in affinity for hirudin$^{54-65}$ peptides despite subtle differences in the exosite environments. Removal of F2 from the proteinase domain thus does not affect the affinity of exosite I for the specific peptide ligand. Previous studies indicated no detectable binding of thrombomodulin to exosite I on human Pre 2 (31), whereas fluorescein-labeled hirudin$^{53-64}$ (hirugen) bound with the same high affinity to bovine Pre 2 as to bovine thrombin (32). The source of this difference is not known but may be due to intrinsic differences in the affinities for human and bovine Pro species (26).

On the Pre 1 activation pathway, the activating cleavage in the proteinase domain at Arg$^{320}$-Ile$^{321}$ to give MzT(-F1) resulted in an 11-20 fold increase in affinity for hirudin$^{54-65}$ peptides. This result was indistinguishable from the 13-20 fold increase in affinity seen on conversion of Pre 2 to thrombin, supporting the conclusion that cleavage in the proteinase domain is accompanied by activation of exosite I, in concert
with activation of the proteinase catalytic site. Thrombomodulin and an exosite I-specific antibody (35), and fluorescein-labeled hirugen (32) have been shown to bind MzT(-F1) with moderate to high affinity, consistent with expression of exosite I on this activation intermediate. Previous results with fluorescent hirugen and bovine proteins, however, indicated 2-8 fold higher affinity for MzT(-F1) compared to thrombin (32), which was not observed in our studies.

Exosite I is disordered in unliganded bovine thrombin and Pre 2, and likely human Pre 2, and becomes structured on the binding of hirudin peptides (46, 47). Comparison of the X-ray crystal structures of hirugen bound to human Pre 2 and thrombin shows that the structures of the exosite-bound peptides are the same, providing no explanation for the differences in affinity observed here between Pre 2 and thrombin (46). The main difference between Pre 1 and Pre 2, thrombin and MzT(-F1) is correct folding of the “activation domain”; chymotrypsinogen segments 10-19 around the Arg15-Ile16 cleavage site, 142-152, 186-194, and 216-226 (48-50). Our results indicate that the affinity of hirudin peptides for exosite I is linked to the folding of segments that activate the catalytic site. Like chymotrypsinogen (49), the activation domain of human Pre 2-hirugen complex is structured in the zymogen but in different conformations than those in human thrombin-hirugen (46) and bovine MzT(-F1) (51). This is unlike the situation for bovine Pre 2, where the activation domain is disordered in the zymogen (47), as is the case for trypsinogen (48). This structural difference between human and bovine zymogens may be relevant to the differences in exosite expression observed here and in previous studies of bovine Pro species (32). Our results suggest that the structures of the activation domain in human Pre 2 and Pre 1 may stabilize the low
affinity state of proexosite I on the zymogen species, which is alleviated when the activation domain is folded correctly into the active conformation.

Although the autolysis loop (Glu146-Lys149e) is part of the thrombin activation domain, it exhibits somewhat more independent properties. The loop is more flexible in the human thrombin-hirugen structure compared to the Pre 2 complex (46), suggesting a possible role for mobility of this loop in the expression of exosite I affinity. The autolysis loop is also defined in the structure of a complex of thrombin with a platelet receptor peptide, but is in a different conformation from that seen in the Pre 2-hirugen complex (46, 52). On the other hand, the autolysis loop in bovine MzT(-F1) is highly structured in the absence of peptide (51), while the exosite I affinity determined here for human MzT(-F1) is the same as that for thrombin. With the information presently available it is not clear whether changes in the structure of the autolysis loop play a role in the affinity of hirudin peptides for (pro)exosite I.

The possibility that exosite II was altered during Pre 1 activation was investigated for the first time by comparison of F2 binding to Pre 2 and thrombin. F2 bound to Pre 2 and thrombin with affinities differing by ~2-fold, indicating that exosite II is not altered significantly by proteolytic cleavage at Arg320-Ile321 to activate the catalytic site. The dissociation constant of [5F]Hir^{54-65}(SO_{3}^-) for Pre 2 was not affected by near-saturating levels of F2, indicating that interactions of these exosite I and II ligands are not allosterically linked on Pre 2. The absence of significant thermodynamic linkage between exosites I and II on Pre 2 is consistent with the previous finding that F2 binding to exosite II on thrombin did not alter hirudin^{54-65} binding to exosite I (45). The present results imply that exosite II is not activated conformationally during conversion of Pre 2
to thrombin but is unmasked by passive dissociation of the F2 domain accompanying cleavage at Arg^{271}-Thr^{272}.

The 11-20 fold increase in hirudin peptide affinity on conversion of Pre 1 to MzT(-F1) was essentially the same as seen for conversion of Pre 2 to thrombin, independent of F2. The overall ∼100-130 fold increase affinity of hirudin^{54-65} previously demonstrated for Pro and thrombin (26) can be approximately accounted for by the ∼6-fold increase in affinity upon removal of F1 from Pro and the ∼20-fold increase in affinity upon proteolytic cleavage of Pre 1 at Arg^{320}-Ile^{321}. The results indicate that peptide bond cleavage at Arg^{320}-Ile^{321} activates exosite I, giving rise to its increased affinity for binding of substrates, inhibitors, and regulatory molecules. Although exosite I on MzT(-F1) and thrombin has equal affinity for hirudin^{54-65}, the exosite I-dependent macromolecular substrate specificities are different. MzT and MzT(-F1) have only 2-12% of the exosite I-dependent activity of thrombin in cleaving fibrinogen, activation of platelets, and activation of factor V in the absence of membranes (53-55). Both MzT and MzT(-F1), however, bind thrombomodulin with affinity comparable to thrombin and exhibit comparable activity in protein C activation (53, 55), which are also exosite I-dependent processes. Similarly, MzT and MzT(-F1) react with the exosite I-dependent inhibitors, hirudin (55) and heparin cofactor II (56), and MzT(-F1) binds an exosite I-specific antibody (31). The low activity of MzT and MzT(-F1) in factor V activation may reflect the participation of exosite II, which is inaccessible on MzT and MzT(-F1), in addition to exosite I (19-21). The X-ray crystal structure of bovine MzT(-F1) suggested that the altered orientation of F2 in MzT(-F1) compared to F2 in the non-covalent thrombin:F2 complex could result in an inhibitory interaction of F2 with fibrinogen (51), which could
also affect factor V activation. The novel conformation of the autolysis loop in MzT(-F1) has also been suggested to inhibit the interaction with fibrinogen (51). In the case of fibrinogen, however, previous studies have found that the active site-blocked human thrombin-F2 complex and MzT(-F1) bind fibrinogen through exosite I with only 2-4-fold lower affinity than thrombin (45). Together, the above results indicate that access of a variety of macromolecules to exosite I on MzT(-F1) is only modestly restricted. On the basis of the disorder of exosite I in free thrombin, binding of exosite ligands is thought to induce structural rearrangements in exosite I to optimally accommodate different ligands. Consistent with this idea, functional mapping of specific interactions of thrombin involved in fibrinogen clotting (15), factor V activation (22), thrombomodulin binding (15), and platelet receptor peptide cleavage (58) has shown that these processes involve different overlapping subsets of residues within exosite I. Whether differences in subset interactions induced in MzT(-F1) and thrombin contribute to the altered substrate specificity of MzT(-F1) is not known. Other structural differences between MzT(-F1) and thrombin outside exosite I may also be involved in fibrinogen substrate recognition, such as changes in the active site substrate specificity associated with the different position of the 60(Tyr-Pro-Pro-Trp)-loop seen in MzT(-F1) (51), and differences in the apolar binding site (57, 59). Thus, the sources of the differences in macromolecular substrate specificity are not completely understood but may reflect differences in binding and catalysis of substrate cleavage, the additional role of exosite II in some of the interactions, and structural differences among MzT(-F1), thrombin, and the thrombin-F2 complex.
Previous studies indicate that (pro)exosite I plays an important role in factor Va-mediated Pro and Pre 1 substrate recognition by the factor Xa-factor Va complex, possibly by mediating productive binding directly (27, 29, 30). The present results showing differential expression of increased exosite I affinity on the Pre 1 activation intermediates suggest that (pro)exosite I-mediated interactions may regulate the factor Va-substrate affinities and rates of cleavage of the activation intermediates at the two sites required to form thrombin. Expression of increased exosite I affinity on MzT(-F1) and not on Pre 2·F2 complex may thus contribute to the cofactor activity of factor Va to redirect the activation pathway from Pre 2·F2 through MzT(-F1). Such changes in (pro)exosite I-mediated Pro-factor Va complexes may reorient the reaction intermediates bound to the factor Xa-factor Va catalytic complex for more optimal cleavage of either of the two sites.

The unexpected, increased affinity of hirudin54-65 on conversion of Pro to Pre 1 observed here suggests that removal of F1 from Pro is associated with a conformational change and/or that the F1 domain directly interferes with binding of the peptides to Pro. The companion paper explores the role of activation fragment 1 in modulating exosite I activation during Pro activation by factor Xa.

ACKNOWLEDGEMENTS

The excellent technical assistance of Jennifer L. Ray, Lori Ray-Cox, and Sarah Tomlinson is gratefully acknowledged.
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FOOTNOTES

*This work was supported by National Institutes of Health Grant HL38779 to P.E.B.
P.J.A. was supported by a postdoctoral fellowship from the American Heart Association,
Southeastern Consortium (SE-9820133V) and subsequently by an Institutional National
Research Service Award (DK-07186).

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1. The abbreviations used are:
Pro, prothrombin
Pre 1, prethrombin 1
Pre 2, prethrombin 2
F1, fragment 1
F2, fragment 2
MzT, meizothrombin
MzT(-F1), meizothrombin des-fragment 1
T, thrombin
FPR-CH₂Cl, D-Phe-Pro-Arg-CH₂Cl
Hir₅⁴-₆⁵(SO₃⁻), Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO₃⁻)-Leu-Gln
[5F]Hir₅⁴-₆⁵(SO₃⁻), Hir₅⁴-₆⁵(SO₃⁻) labeled at the amino terminus with 5-
carboxy(fluorescein)
hirugen, hirudin₅³-₆⁴
Figure 1. Fluorescence excitation difference spectra of [5F]Hir^{54-65}(SO_3^-) binding to prethrombin 1, its activation intermediates, and thrombin. Fluorescence excitation difference spectra (ΔF) are shown for 0.2 µM [5F]Hir^{54-65}(SO_3^-) binding to 5 µM Pre 1 (green) and 7 µM Pre 2 (blue), representing 91% and 94% saturation, respectively. Also shown are fluorescence difference spectra for 0.2 µM [5F]Hir^{54-65}(SO_3^-) binding to 1 µM thrombin (red) and 0.5 µM MzT(-F1) (black), at 97% and 93% saturation, respectively. Spectra were collected and analyzed as described in Experimental Procedures.

Figure 2. Quantitative affinity chromatography of Pre 1 on Hir^{54-65}(SO_3^-)-agarose. The absorbance (A_{230nm}) elution profiles are shown for chromatography of 6.7 µM Pre 1 (0.1 mg) in l 0.15 M, pH 7.4 buffer on Hir^{54-65}(SO_3^-)-agarose (□), in buffer containing 2 M NaCl (○), or on a control column lacking peptide (●). Results are also shown for chromatography of Pre 1 in the presence of Hir^{54-65}(SO_3^-) at 0.5 µM (■), 1 µM (▲), or 4 µM (▲). Inset, the fractional change in elution volume ((V_{obs}-V_o^*)/V_o^*) as a function of total hirudin^{54-65} peptide concentration (|Hir^{54-65}(SO_3^-)|) and the fit by the hyperbolic equation with the parameters described in the text. Chromatography was performed and analyzed as described under Experimental Procedures.

Figure 3. Direct and competitive titrations of [5F]Hir^{54-65}(SO_3^-) and Hir^{54-65}(SO_3^-) binding to Pre 1 in the absence and presence of F1. A. The fractional change in fluorescence (ΔF/F_o) of 50 nM [5F]Hir^{54-65}(SO_3^-) shown as a function of total Pre 1
concentration ([Pre1]₀) in the absence (●) and presence (○) of 30 µM F1. B. Fluorescence changes of 50 nM [5F]Hir⁵⁴⁻⁶⁵(SO₃⁻) and Pre 1 at concentrations of 1µM (●) and 3 µM (○) as a function of the total concentration of unlabeled hirudin peptide ([Hir⁵⁴⁻⁶⁵(SO₃⁻)]₀). The lines represent the nonlinear least-squares fits of the quadratic (A) and competitive (B) binding models with the parameters given in Table I. Titrations were performed and analyzed as described under Experimental Procedures.

Figure 4. Fluorescence titrations of [6F]FPR-thrombin with F2 in the absence and presence of competing Pre 2. The fractional change in fluorescence of 190 nM [6F]FPR-thrombin as a function of total fragment 2 concentration ([F2]₀) in the absence (●) and presence (○) of 20 µM Pre 2. The lines represent the nonlinear least-squares fits of the competitive binding model with the parameters described in the text. Titrations were performed and analyzed as described under Experimental Procedures.

Figure 5. Direct and competitive titrations of [5F]Hir⁵⁴⁻⁶⁵(SO₃⁻) and Hir⁵⁴⁻⁶⁵(SO₃⁻) binding to Pre 2 in the absence and presence of F2. A. Fluorescence changes of 50 nM [5F]Hir⁵⁴⁻⁶⁵(SO₃⁻) as a function of total Pre 2 concentration ([Pre2]₀) in the absence (●) and presence (○) of near-saturating F2 (20 µM). B. Fluorescence changes of mixtures of 50 nM [5F]Hir⁵⁴⁻⁶⁵(SO₃⁻) and 0.5 µM (▲) or 2 µM (△) Pre 2 as a function of total unlabeled hirudin peptide concentration ([Hir⁵⁴⁻⁶⁵(SO₃⁻)]₀). The lines represent the nonlinear least-squares fits of the ternary complex model (A) or competitive binding model (B) with the parameters given in Table I. Titrations were performed and analyzed as described under Experimental Procedures.
Figure 6. Direct and competitive titrations of [5F]Hir^{54-65}(SO_3^-) and Hir^{54-65}(SO_3^-) binding to MzT(-F1). A. Changes in fluorescence of 50 nM (●) or 250 nM (○) [5F]Hir^{54-65}(SO_3^-) are shown as a function of total MzT(-F1) concentration ([MzT(-F1)]_o).

B. The fractional changes in fluorescence of mixtures of 50 nM [5F]Hir^{54-65}(SO_3^-) and 100 nM (●) or 300 nM (○) MzT(-F1) shown as a function of total concentration of unlabeled hirudin peptide ([Hir^{54-65}(SO_3^-)]_o). The lines represent the nonlinear least-squares fits of the quadratic (A) or competitive (B) binding models with the parameters listed in Table I. Titrations were performed and analyzed as described in Experimental Procedures.
Table 1

Parameters determined for binding of hirudin\textsuperscript{54-65} peptides to prethrombin 1, the prethrombin 1 activation intermediates, and products.

The dissociation constants ($K_D$), stoichiometric factors ($sites$), and maximum fluorescence changes ($\Delta F_{max}/F_0$) determined for hirudin\textsuperscript{54-65} peptides from direct and competitive titrations, and ternary complex analysis are listed. Titrations were performed and data analyzed as described in Experimental Procedures.

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Method</th>
<th>$K_D$ ($\mu M$)</th>
<th>Sites (mol/mol protein)</th>
<th>$\Delta F_{max}/F_0$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[5F]Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Direct Titration</td>
<td>3.2±0.3</td>
<td></td>
<td>-17.6±0.5</td>
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<tr>
<td>Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Competitive Titration</td>
<td>2.6±0.6</td>
<td></td>
<td>-18.7±0.4</td>
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<tr>
<td>Prethrombin 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[5F]Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Direct Titration</td>
<td>0.5±0.1</td>
<td></td>
<td>-21.1±0.5</td>
</tr>
<tr>
<td>Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Competitive Titration</td>
<td>0.7±0.1</td>
<td></td>
<td>-21.1±0.5</td>
</tr>
<tr>
<td>+F1: [5F]Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Direct Titration</td>
<td>0.6±0.1</td>
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<td>-19.3±0.4</td>
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<td>Quantitative Affinity</td>
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<td>Chromatography</td>
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<td>Prethrombin 2</td>
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<td></td>
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<tr>
<td>[5F]Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Direct Titration</td>
<td>0.44±0.04</td>
<td></td>
<td>-26.5±0.5</td>
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<tr>
<td>Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Competitive Titration</td>
<td>0.5±0.1</td>
<td></td>
<td>-26.6±0.8</td>
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<tr>
<td>+F2: [5F]Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Ternary Complex</td>
<td>0.4±0.1</td>
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<td>-25.8±0.7</td>
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<td>Meizothrombin des-fragment 1</td>
<td></td>
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<td></td>
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<tr>
<td>[5F]Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Direct Titration</td>
<td>0.024±0.003</td>
<td>1.1±0.1</td>
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<tr>
<td>Hir\textsuperscript{54-65}(SO$_3^-$)</td>
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<td>0.065±0.013</td>
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<td>-31.4±1.0</td>
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<td>Thrombin</td>
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<td></td>
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<tr>
<td>[5F]Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Direct Titration</td>
<td>0.025±0.002</td>
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<tr>
<td>+F2: [5F]Hir\textsuperscript{54-65}(SO$_3^-$)</td>
<td>Ternary Complex</td>
<td>0.020±0.007</td>
<td></td>
<td>-27.6±0.7</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3

\[ \frac{\Delta F}{F_0} \]

\[ [\text{Pre 1}]_o \mu M \]

\[ [\text{Hir}^{54-65}(\text{SO}_3^-)]_o \mu M \]
Figure 4
Figure 6
Effects of activation peptide bond cleavage and fragment 2 interactions on the pathway of exosite I expression during activation of human prethrombin 1 to thrombin

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J. Biol. Chem. published online August 25, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306917200

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